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CAUSATIVE FACTORS AND A THERAPEUTIC APPROACH

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Mava Nash

PI Signature

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INTRODUCTION

Laser exposure of the eye is harmful and may cause an immediate vision loss, when the macula is affected. Our previous work (1-7) has demonstrated the major role played by inflammation in lasered eyes as indicated by enhanced levels of various pro-inflammatory indicators, such as prostaglandins and leukotrienes; The inflammatory laser processes extend over an area greater than the laser lesion site as shown histologically.

Most laser ocular injuries affect the retinal area away from the macula.

Those retinal injuries caused by low-level energy laser exposure might be initially symptomless, but they evolve later (weeks to months), due to fibroproliferative reaction, into scarring, leading to severe visual impairment. Our study investigated scar formation, occurring at the vitreoretinal interface and/or extending into the choroid. Scarring is also associated with an enhanced angiogenic activity or neovascularization, a most fearful complication in the eye leading to bleeding and/or retinal detachment. So far, the role of vitreous in eyes subjected to laser retinal injury has not been studied. Additionally, no treatment has been suggested to prevent or decrease laser injury in the eye.

Our work is divided into three sections (A-C) as follows;

1. Effect of vitreous from lasered eyes on cell proliferation of Retinal

Pigment Epithelium, cells Vascular Endothelium cells and fibroblasts in cells culture.

2. The angiogenic effect of lasered vitreous and the effect of various anti-inflammatory drugs and antioxidants on the angiogenic activity.
3. Laser-induced ocular scarring - involvement of inflammation and neovascularization;

A. Effect of vitreous from lasered eye on cell proliferation

Intraocular cellular proliferations of Retinal Pigment Epithelium (RPE) and of fibroblasts are the main components of intravitreal and subretinal membranes and scarring (8-10), and they are involved in many disorders such as diabetic retinopathy, and scarring post surgery or post laser.

Intravitreal and subretinal scarring by RPE cells and fibroblasts is termed Proliferative Vitreo Retinal (PVR) disorder.

PVR occurs after RPE cells and fibroblasts gain access to the vitreal cavity, in which they are exposed to the vitreous humor, undergo proliferation, deposit collagen and form fibrotic membranes. These events are often preceded by macrophage infiltration into the vitreous (8,11,12) that may also influence cellular proliferation.

The nature of the factor/s stimulating vitreo retinal proliferation is not known, nor is it known whether such a factor is derived primarily from the vitreous, from the macrophages or from both. Macrophages secrete substances which are mitogenic for fibroblasts in vivo (13,14) and in vitro (42,43), as well as for RPE cells (17).

The vitreous provides also a very permissive milieu for the proliferation of many cell types in vivo (9,18) and for RPE cells in vitro (19).

In order to find out whether vitreous bodies from eyes subjected to retinal laser exposure, termed "lasered vitreous", cause proliferative changes, we established a full scale dose response curve for the proliferative effect of vitreous on three cell lines: RPE cells, vascular endothelial cells and fibroblasts.

B. The angiogenic effect of lasered vitreous and various preventive treatment modalities

The interaction of the retinal pigment epithelium (RPE) with the vascular endothelium determines the resultant fibrovascular response, involved with scarring and with associated visual impairment. Angiogenesis occurs in response to various pathological and physiological stimuli, and incorporates a complex array of biological events, including: activation of vascular endothelial cells (VEC), breakdown of basement membranes, VEC migration, VEC proliferation, lumen formation and secretion of new basement membrane (20). The regulatory mechanisms responsible for these processes are not completely understood.

The list of factors shown to induce "angiogenic activity" in the eye and elsewhere in the body include: 1) Acidic Fibroblast Growth Factor (aFGF); 2) Basic Fibroblast Growth Factor (bFGF); 3) Angiotensin; 4) Transforming Growth Factor (TGF, alpha, beta (10); 5) Tumor Necrosis Factor (TNF); 6) Prostaglandin (PGE_1 , PGE_2 and prostacyclin; 7) Low molecular weight factors; 8)

Chemotactic factors; 9) Modulating factors (Heparin, Copper) (21).

RPE plays a multifanious role in ocular neovascularization and scar formation, and its interaction with endothelial cell proliferation warrants special consideration. Dual effect of RPE on endothelial cell proliferation is still controversial. Thus, both stimulation (22) and inhibition (23-24) of vascular endothelial cell growth by RPE are determined by different environmental conditions (25,26). The close interaction of RPE and vascular endothelium was further emphasized by the observation that RPE cells migrate into the area of laser-induced subretinal neovascularization and surround the newly formed vessels, suggesting the release of chemoattractant factors for RPE by the endothelial cells, an observation which was later confirmed by Campochiaro and Glaser (27,28).

Both proliferation and migration activities of RPE can be stimulated by factors released from macrophages (29,30), indicating that RPE response is enhanced during the inflammatory reaction.

Although the involvement of the "extracellular modulating factors" in determination of the intraocular neovascularization is well-established (18), data on their role in the development of laser-induced scar formation and neovascularization are scarce and contain data on bFGF and TGF-beta in healing laser lesions (already discussed) (31,32). We will, therefore, try to evaluate the contribution of lasered vitreous on angiogenesis in vivo, using Fournier's

technique on rat's cornea.

The drugs studied to reduce and/or prevent angiogenesis were:

- 1) A non steroidal anti inflammatory drug, aimed at inhibiting Leukotrienes' production - NorDihydroguaiaretic acid (NDGA).
- 2) An antioxidant, reducing the NADPH activity - Diphenylene Iodonium (DPI).
- 3) A non steroidal antiinflammatory drug - Indomethacin.
- 4) A steroid - Dexamethasone.

The use of steroidal and nonsteroidal anti-inflammatory drugs in various intraocular proliferative disorders has already been suggested (33,34). When tested for their ability to inhibit proliferation of various cell cultures, it was found that addition of various nonsteroidal anti-inflammatory drugs to rat hepatoma and human fibroblast cultures induced a reversible inhibition in cell proliferation (35). Indomethacin was found most potent. Steroidal anti-inflammatory drugs possess similar antiproliferative properties, as has been demonstrated experimentally following intravitreal infections (33). Additionally, neovascularization associated with membrane formation was also significantly reduced (33,34) by this drugs. However, paradoxically, at high concentrations steroids stimulated proliferation of dermal fibroblasts (34). Similarly, steroids have a biphasic effect on prostaglandins (PGs) production, as demonstrated by us (5-7); the steroidal inhibitory effect is transitory, and limited to the initial post-laser phase.

In addition to anti inflammatory drugs, an antioxidant inhibiting NADPH system (DPI) was studied in this work because of the role played by "free radicals" in inflammation.

C. Laser-Induced Ocular Scarring - Involvement of Inflammation

Neovascularization at the vitreoretinal interface (retinal scarring) is of the most feared complications following laser retinal exposure, leading to retinal tears, atrophy and a severe visual loss.

Subretinal neovascularization as a complication in patients subjected either to Argon or to Krypton laser treatment (37-41), or in people accidentally exposed to industrial laser (31-33), has rarely been observed. Experimentally it appeared during the first few weeks post-laser and regressed in an average of 2.2 months but resulted in severe visual impairment due to heavy scarring and retinal wrinkling (31,33).

The role of inflammation in ocular neovascularization has been confirmed in some studies. Retinal detachment in cats resulted in neovascularization of the iris (15); aqueous obtained following ocular injury (paracentesis) contained excessive levels of PGs and stimulated bovine corneal endothelium proliferation in culture (16).

Data inferring that PGs and leukotrienes (known for their pivotal modulating effect in the inflammatory reaction) are also involved in angiogenesis, wound repair and fibroproliferative disorders in various organs, have been well

documented; the observation of the neovasculogenic ability of PGE_1 and PGE_2 in rabbit's cornea is well established (18,19). The neovasculogenic activity of an anti-lenkotriene drug - NDGA - given concomitantly with steroids will be studied as well because of the close involvement of lenkotrienes in inflammation.

A. Vitreous from lasered eyes - effect on cell proliferation

Materials and Methods

Vitreous humor collection

Vitreous humor collection from lasered and non lasered (intact) eyes was done as previously described (4,7). In brief, the cornea was cut at the limbus and then removed, followed by removal of the lens and iris. The vitreous was then gently pulled and its attachments at the posterior segment were carefully cut, so that it was released.

The vitreous body was homogenized with a Potter-Elvehjem tissue homogenizer, centrifuged at $10.000\times g$ for 10 minutes at 4°C , filter sterilized and stored at -70°C .

Culturing of retinal pigment epithelium

The RPE cells were harvested from human eyes obtained within 72 hours post mortem from Cornea Bank Shiba Medical Center in Tel-Aviv.

The anterior segment including the cornea, iris and lens were removed

following a circumferential cut through the pars plana just anteriorly to the ora serrata. The vitreous was next aspirated without damaging the retina and the retina was gently separated from the RPE cell layer with fine scissors. The eye cup was then washed in MEM, trypsinized (0.25% trypsin/ 0.05% EDTA, Irvine Scientific) and incubated at 37°C for 45 minutes. Primary cultures were established by gently pipetting the cell suspension in the eye cup into a 25 cm² tissue culture flask coated with fibronectin and incubating the flask at 37°C in an atmosphere of 5% co2/95% air. The growth medium consisted of Ham F-10, supplemented with 15% fetal calf serum, 0.4% glucose, gentamicin 40 mg/ml (gentamicin sulfate solution, Irvine Scientific) and L-glutamine 1% (L-Glutamine 200mM, 29.2 mg/ml, Irvine Scientific). Within 7-14 days of seeding in the culture medium a proportion of cells were attached and showed a flattened morphology. 10 days following attachment, cell division began. The cells were subcultured on reaching confluency. The purity of cells was checked under a phase contrast microscope. Only cultures that exhibited a homogeneous population of darkly pigmented polygonal cells were processed further, and third to fourth passage cells were used in all experiments.

Human vascular endothelium culturing - Human vascular endothelium was

purchased from American type culture collection, 12301 Parklawn Drive Rockville MD. 20852 ,U.S.A.

The endothelial cells were grown in 55cm² plates containing Dulbecco minimal essential medium (DMEM) supplemented with 5% bovine serum, 5% fetal calf serum, 1% L-Glutamin and antibiotics in 5% CO₂/95% air at 37°C. Fourth to fifth passages of cells were used for experiments.

Human fibroblast skin culture - fibroblasts which were purchased from American type collection (as described) were grown in 55 cm² plates containing DMEM supplemented with 10% fetal calf serum, 1% glutamine and antibiotics in 5% CO₂ 95% air at 37°C. Cells at passages 10 through 16 were used for experiments.

³H-Thymidine uptake - This assay was done following a well established technique. In brief, RPE cells at confluence were lightly trypsinized and plated in 16 mm wells of 24-well plate at 2×10^4 cells per well in nutrient mixture Ham's F-10 supplemented with fetal calf serum. The cells were grown for two days till confluency, then they were washed and the medium was changed to serum free-medium, containing either vitreous or a growth factor according to protocol. After 18 hours of incubation the cells were pulsed with 2 UCi/ml of ³H-Thymidine (6.7 Ci/m Mol; New England) for 2 hours. The cells were washed three times with phosphate buffered saline and five times with ice cold 5% trichloroacetic acid. One ml of 0.1 NAOH containing 0.1% sodium dodecyl sulfate was then added to each well, and after one hour a 100 ul aliquot was added to 4.5 ml of Scintillation fluid and counted in a Beckman Scintillation counter (Irvine Ca).

Experimental design:

The experiments involved three cell lines: 1) RPE cells; 2) Vascular endothelial cells; 3) Human skin fibroblasts, and therefore a set of experiments was performed for each cell line; Experimental design for RPE and vascular endothelium was similar and involved 8 groups for each cell line as follows:

Group 1 - control - cells grown in medium with no vitreous added.

Groups 2-4 - Vitreous control - cells grown in conditioned media containing non-lasered vitreous diluted to concentrations of 5%, 15% and 25%.

Groups 5-7 - Lasered Vitreous - cells grown in conditioned media containing three concentration of lasered vitreous: 5%, 15% and 25%.

Group 8 - Epidermal growth factor - cells grown in media containing EGF (3ng, ml).

The study of human skin fibroblasts involved 14 groups:

Group 1 - control - cells grown in conditioned media containing no vitreous.

Group 2-7 - Vitreous control - cells grown in conditioned media containing non-lasered vitreous at concentrations of 1.25%, 2.5%, 5%, 10%, 20% and 25%.

Groups 8-13 - Lasered vitreous - cell grown in conditioned media containing lasered vitreous at the following concentrations: 1.25%, 2.5%, 5%, 10%, 20%, 25%.

Group 14 - bFGF group - cells grown in conditioned media containing basic fibroblast growth factor (10 ng / ml).

During the first three days following seeding, the cells were grown (as described) in a conditioned medium free of vitreous or growth factors. Later, when confluency was as yet not achieved, the medium was changed according to protocol into medium containing either lasered vitreous, non-lasered vitreous or a growth factor for a 24 hour incubation period. When confluency was achieved the medium was again changed and Thymidine uptake was determined (as described).

The vitreous from lasered or non-lasered eyes was diluted according to protocol with the appropriate conditional medium.

Proliferation assay

Each control or experimental group consisted of 16 parallel culture.

Fibroblasts, RPE cells and vascular endothelium cells were plated in a 48 well cluster plates (Costar Cambridge Ma. USA) at a density of 10,000 (for fibroblasts and vascular endothelium) and 2×10^4 (for RPE) and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal calf serum. After a period of two days to allow attachment and initial proliferation, the medium was removed, the cells were washed with serum-free DMEM and then vitreous at various concentrations was added for an incubation period of 24 hours.

To establish a dose response curves, vitreous was dibuted to final concentrations of 1.25%, 2.5%, 10% and 25% in medium containing 5% serum. Each experimental group was compared to a control group with an equal number of cells.

Following a 24 hour incubation period with the vitreous or control conditioned media, Thymidine uptake assay was performed.

Results

Vascular endothelium proliferation

Vitreous obtained from intact eyes and applied at concentration of 5%, 15% and 25% to growth medium had a negligible effect on Thymidine uptake of vascular endothelium cells when compared to control (Fig. 1). However, addition of bFGF increased vascular endothelial proliferation by 1.5 when compared to control. Lasered vitreous at the same concentrations did not have a significant effect on cell proliferation, either. It occurred to us that lasered vitreous might contain cytokinins (such as prostaglandins, leukotrienes, interleukins) at such high levels in which they might induce noxious stimuli, and thus curtail cell proliferation. Therefore, the experiments were repeated using the following concentrations of lasered vitreous: 1.25%, 2.5%, 5%, 10%. As indicated in Fig. 2, using lasered vitreous at concentration of 1.25% and 2.5% induced a two-fold increase in cellular proliferation for both concentrations as compared to control.

Retinal pigment epithelium proliferation

Intact vitreous caused an induction of RPE cell proliferation as manifested by enhanced Thymidine uptake in a dose related manner, as shown in Fig. 3.

A maximal 2.5 - fold increase was exerted at 25% concentration of vitreous, when compared to control.

Lasered vitreous also affected Thymidine uptake by RPE cells in a dose related manner and was found to be more effective than the vitreous taken from intact eyes.

EGF caused only a negligible increase (1.4 - fold) in Thymidine uptake, when compared to control.

Fibroblast proliferation

Vitreous from intact eyes caused a dose related increase (Fig. 4) in fibroblast proliferation, with maximal effect at concentration of 10%, in which the Thymidine uptake was 2.8 fold higher than control values. With 20% concentrations of intact vitreous, Thymidine uptake was 50% greater than control, and at 25% concentration control values were resumed .

Lasered vitreous also caused a dose related increase in Thymidine uptake of fibroblasts, but to a lesser degree than the intact vitreous (Fig. 4). Thus the maximal Thymidine uptake of 2 fold higher than control was achieved at 5% and 10%, as compared with maximal enhanced Thymidine uptake of 2.8 with the intact vitreous.

Discussion

In this study the three cell types studied-fibroblasts, vascular endothelium and RPE cells take place in intraocular scarring, e.g. proliferated vitreo retinopathy.

Our finding showed that nonlasered vitreous had a negligible effect on vascular endothelial proliferation while it enhanced Thymidine uptake in RPE cells and in fibroblasts in a dose related manner. Fibroblasts were affected by lower concentration (10%) of vitreous which induced greater cell proliferation than its effect on RPE cells (maximal at 25%). This finding indicates that non lasered vitreous effect on cell proliferation is cell specific and dose related. Lasered vitreous enhanced Thymidine uptake of the three cell lines in a dose related manner with maximal effectiveness at concentration of 10%, 2.5% and 25% for fibroblasts, vascular endothelium and RPE, respectively. However, lasered vitreous effect on fibroblast proliferation was to a lesser degree than that of the non lasered vitreous. Thymidine uptake of RPE and vascular endothelium was enhanced to a greater extent by lasered vitreous when compared to non lasered vitreous. Vascular endothelial susceptibility to lasered vitreous was greater than that of RPE. Thus, enhancement of Thymidine incorporation by endothelial cells occurred at vitreous concentration of 2.5%, comparing to 25% with RPE cells.

Our results concerning the proliferative activity of non lasered vitreous on

RPE is in agreement with report on similar dose related proliferative activity on RPE and fibroblasts in human eyes (44).

Our finding of a mitogenic activity of lasered vitreous on vascular endothelium is in agreement with a report on the proliferation of bovine retinal microvascular endothelial cells in basal medium containing lasered vitreous of mini pigs in a concentration of 1:20 (45). In this study non lasered vitreous was also mitogenic for endothelial cells, using basal conditioned media.

The finding on vitreal effect in RPE cells might be explained by a report on RPE cell culture showing that RPE cells respond to endogenous vitreous exposure by modifying secretion and cell surface glycoprotein expression. These biochemical changes corresponded temporally to transformation of the epithelial cell pattern to an elongated fibroblast morphology (46).

The dual effect of lasered vitreous on vascular endothelial proliferation, with proliferation inhibition in higher vitreal concentrations and an enhanced

Thymidine uptake following dilution of vitreous, might be related to endothelial susceptibility to cytokinins present in the vitreous, such as prostagladins, leukotrienes and interleukins that might act as toxic stimuli; This duality might also be related to the presence of stimulators or inhibitors in the vitreous humor which are released by RPE and affect vascular endothelium (47).

Our finding that lasered vitreous had no enhancing effect of Thymidine uptake

of fibroblasts when compared to non lasered vitreous might be explained by the fact that vascular mitogenic activities in the vitreous and retina have no effect on fibroblasts and several other cell lines including rabbit corneal epithelial cells (48) and Keratocytes.

Following laser retinal damage the retina and RPE are activated to release various growth factors, stimulators and cytokinins. It seems from our work that fibroblasts are unaffected by these factors, while vascular endothelial cells show great susceptibility to relatively small amounts of the various factors associated with cell proliferation.

The role of arachidonic acid metabolites, both leukotrienes which are the lipoxygenase products and prostaglandins which are the cyclooxygenase products, in cellular proliferation and in angiogenesis is well established (Wilson).

It has been shown that retinal laser damage is associated with elevated levels of prostaglandins and leukotrienes in the vitreous. These eicosanoids might affect differentially cellular proliferation, as indicated previously by the differential effects of indomethacin on the growth of RPE, fibroblast like cells and vascular endothelial cells. A varied concentration of Indomethacin was required to inhibit proliferation of each cell line (Berman).

B. The angiogenic effect of lasered vitreous - therapeutic trials.

This work involved studies on the efficacy of known antiinflammatory agents used by us in the past, such as NDGA (an antileukotriene agent) and steroids, as well as a new drug, an NADPH oxidase inhibitor, termed D.P.I. (Diphenylene Iodomium). DPI is known for its ability to reduce superoxidase formation. The study involved induction of angiogenesis in rat's cornea by pellets containing vitreous of lasered eyes from DPI was incorporated into the pellet and was not administered systemically because of a problem in forming an ethanol-free solution. However, presently we have overcome this problem and we possess an ethanol-free DPI which can be administered intramuscularly.

Materials & Methods

Laser procedure of rabbits - Rabbits have been exposed to 30 burns by Nd:YAG laser, following a protocol already used by us during the last few years in our US Army grants.

Vitreous sample preparation - In the enucleated eyes the cornea was cut at the limbus, and the iris was removed by pulling at its base. Two meridional scleral incisions of the limbus facilitated vitreous separation using fine scissors to free it from its attachment to the retina. The vitreous body of each eye was placed in a separate vial and homogenized at 4°C in a Polytron PT 10-35 homogenizer (Kinematica, GmbH, Littan-Luzern, Switzerland) for 3 minutes and then incubated following the addition of 0.3 ml of Krebs Ringer's Bicarbonate Heppes buffer, pH 7.4, in a slow shaking bath at 37°C for 15 minutes. Portion of the vitreous was used for biochemical analysis and portion was incorporated into the pellet, after a lyophilization procedure (to be described below - pellet preparation).

Corneal angiogenesis in rats: Our model of corneal neovascularization was based on Fournier's corneal micropocket assay technique, and involved implantation of pellets containing vitreous extracts of rabbits' eyes into the cornea of fully anesthetized rats. The vitreous was obtained either from lasered eyes or from eyes unexposed to laser, the later serving as control. Corneal neovascularization was assessed clinically and histologically (as described).

Pellet preparation: A weighed quantity of the lyophilized vitreous was mixed with the 10% Elvax (made by Ethylene/vinyl acetate copolymer [Elvax: Aldrich] dissolved in Methylene Chloride after incubation for 30 minutes at 37°C) and vortexed vigorously to produce an homogeneous suspension. A twenty-well Perspex mold was used and the vitreous Elvax suspension was delivered through a pipette and allowed to polymerize at -20°C for 24 hours and then desiccated for 48 hours to evaporate residual solvent. Polymer pellets were cut with a scalpel blade into small pieces 1 ± 0.3 mm² each, with an average weight of 1.5 ± 0.3 mg/pellet.

Pellet implantation: Pellet implantation was performed in fully anesthetized rats. Five minutes prior to the implantation of the pellet, the rat cornea was locally anesthetized with topical application of 0.1% Benoxinate Hydrochloride (Localin: Fischer, Tel-Aviv, Israel). Immediately after the procedure, Neomycin sulfate 0.5% drops (Neocin: Fischer, Tel-Aviv, Israel) were topically applied to the cornea. A mid-corneal incision 3 mm wide, extending into half the depth of the corneal thickness, was made with a No. 65 beaver blade under the operating microscope. A thin surgical cyclodialysis spatula was used to extend the incision into the mid-stroma so that the pellet could be inserted. After pellet implantation, the

micropocket was closed by sliding the corneal epithelium over it with the side of the forceps. All procedures were performed aseptically. Excluded from the study were eyes in which: 1) pellet implantation procedure was prolonged, 2) pellet implantation resulted in corneal perforation, 3) when the pellet was visibly located paracentrally, 4) when expulsion of the pellet took place during the first 4 days, 5) edema was developed on the 1st or 2nd day post surgery.

In vivo assessment of angiogenesis: Rats with implanted pellets were followed up at 2, 5, 7, 14 and 21 days after implantation, under full anesthesia. At each time interval recording of the limbal vascular congestion changes and of corneal neovascularization growing was performed by quantitative microscopy, using the Wild M7S stereomicroscope which can be switched over to give a single vertical beam which ensures parallel-free measurement of the horizontal distance between vertically separated feature. Using of this microscope thus enables measurements of blood vessels growing over the curvature of the cornea and at the same time studying the limbal area whose plan is lower. The measurement is performed using graticules (reticules) which are metal made and easily fitted into any 10x15 and x20 standard adjustable wide-field eye pieces. For length determination of blood vessels a 394771 graticule with a scale of 5 mm:100 was used. The progress of vascularization in our rat model was characterized by an initial congestion of the limbal capillaries at the cornea-limbal area which were enlarged forming loops as described. Subsequently, vascular sprouts appeared at the apices of the loops which later developed into vascular branches growing perpendicular to the limbus into the clear cornea and were directed

toward the centrally located pellet. Limbal congestion and new blood vessel growth were separately assessed. During the first 5 days following implantation the limbal congestion was prominent with only few, tiny new blood vessels growing into the cornea. Starting from day 7, new blood vessels growth became evident while congestion remained unchanged.

Histopathological analysis of corneal neovascularization and inflammatory infiltrate

Assessment of the inflammatory infiltrate at the cornea and at the anterior segment of rats eyes with implanted pellets was done in six randomly assigned rats from each group at 21 days post implantation. The whole eye was fixated in 10% formaldehyde solution for 3 days and was processed in the standard manner for light microscopy, using the Hematoxylin and Eosin staining. Six sections were prepared from each experimental eye, including the iris-ciliary body and the cornea. Six low power (x60) fields were elected randomly across either the ciliary body or the central cornea for assessment of neovascularization and inflammatory response.

EXPERIMENTAL DESIGN

The study involved four groups:

1. Vitreous control - eyes implanted with pellets containing Elvax and vitreous from intact eyes.
2. Pellet control - eyes implanted with pellets containing only Elvax suspension and no vitreous.

3. Lasered pellet - eyes implanted with pellets containing Elvax and vitreous from lasered eyes.
4. DPI treated group - eyes implanted with pellets containing Elvax, lasered vitreous and 10 μ g DPI.

RESULTS

Pellet release of leukotriene B₄ and Prostaglandin E₂

To ensure that a pellet containing vitreous from laser exposed eyes possesses the capability of slowly releasing LTB₄ and PGE₂ into the cornea, the following experiment was done: A total of 14 pellets containing vitreous bodies obtained from either intact (7) or lasered (7) eyes were separately incubated at 37°C in 0.3ml of Krebs Ringer Bicarbonate-HEPES buffer (pH 7.4) in a slow shaking bath for 60 minutes. At the end of incubation period a sample was withdrawn from the medium for PGE₂ and LTB₄ determination. In the media containing pellets with vitreous from intact eyes the levels of either of these metabolites was undetectable. On the other hand, excessive levels of PGE₂ and LTB₄ (1.0 ± 0.2 ng/ml and 1.2 ± 0.2 pg/ml, respectively) were found in the media with pellets containing lasered vitreous. This finding confirms our assumption that the pellet acts as a slow release system, secreting PGE₂ and LTB₄ into the cornea.

Angiogenesis activity

A marked congestion of limbal blood vessels was noted on day 2 post pellet implantation and was similar in all three groups (Fig. 5). Limbal congestion involved a marked vasodilatation of pre-existing veins surrounding the limbal area, but with no new vessels growing into clear cornea. In the pellet and vitreous control groups, on the 5th day post surgery, the limbal congestion subsided and resumed baseline appearance (Fig. 6). Limbal vascular congestion was significantly affected in DPI treated eyes, in which a significant reduction in congestion both on the 2nd and on the 5th day post implantation was noted when compared to untreated laser group or the group treated by combined NDGA and steroids (a combination of two antiinflammatory agents), (Fig 7 & 8).

The mean number of newly formed blood vessels budding was significantly lower in DGI treated group, but not in the other treated group, both on the 5th and on the 12th day post implantation (Fig. 9). The length of corneal neovascularization induced by lasered eyes vitreous was also significantly lowered by DPI on the same days post pellet implantation (Fig. 10).

DISCUSSION

Our work demonstrated that incorporation of the vitreous of lasered rabbit eyes into a copolymer pellet and the latter implantation into a rat's cornea caused severe and prolonged corneal neovascularization. Rat's corneal neovascularization was associated with a significant inflammatory reaction of the cornea and the adjacent ciliary body. The newly formed blood vessels underwent some regression after the pellet was absorbed.

Our finding that prostaglandin and leukotriene levels are elevated in the vitreous of lasered eyes has been previously reported in our prior work. Our demonstration of an angiogenic activity of the lasered vitreous when incorporated into pellets which were implanted into rats' corneas, might be related to the vitreal high content of PGE_2 and/or LTB_4 which are slowly released out of the pellet as demonstrated by us.

However, our data, showing an inhibitory effect of DPI on the angiogenic activity, point to the possibility that superoxide and other free radicals are also released into the vitreous of eyes subjected to laser exposure. For DPI is known as an NADPH oxidase inhibitor. Thus, peroxides may also be involved in the angiogenic process and their inhibition might have a regulatory effect on blood vessel formation.

Our conclusion from our pilot studies is that DPI, which is a superoxide inhibitor, seems to possess an inhibitory effect on laser induced angiogenesis, an effect greater than that observed following treatment by the usual antiinflammatory agents aimed at the arachidonic acid cascade inhibition (NDGA and steroids).

C. Laser induced ocular scarring - the involvement of inflammation.

During this study a pilot work was carried out to study the changes in amounts of prostaglandin E₂ (PGE₂), leukotriene B₄ (LTB₄) and interleukin-1 released by cultured RPE cells into the growth medium following treatment with a growth factor (basic FGF). The working hypothesis is that RPE cells known for their capability to produce the above mentioned cytokinins will eventually release them into the conditioned medium following treatment by bFGF.

MATERIALS AND METHODS

Prostaglandin E₂ determination: PGE₂ was measured using the radioimmunoassay technique with a specific antibody for PGE₂ (Miles-Yeda, Rehovot) which has a 3% cross-reactivity with PGE₁ and less than 1% with other prostaglandins. Day-to-day reproducibility and in-run precision of the analytical procedure was examined by processing, in each assay, samples of vitreous and buffer containing known amounts of PGE₂. To determine PGE₂ recovery rate, the appropriate tritiated prostaglandin was added to the medium, which was then cooled to 4°C, acidified to pH 4 with sodium acetate buffer (1M, pH 3.8) and extracted as described. Part of the dissolved aqueous phase was used to assess procedural loss, and the remainder was used for the radioimmunoassay.

Leukotriene B₄ determination: Leukotriene B₄ was determined using a radioimmunoassay kit (New England Nuclear Research products, New England) with an antibody to leukotriene B₄ with cross reactivity of 3.6% to 5,1 DiHETE and less than 2% to the various lipoxygenase and cyclooxygenase products of arachidonic acid.

Interleukin-1- α was determined using the Elisa technique.

RPE cell culturing - RPE cells were grown in 25 cm² tissue culture flasks (Nunc, Denmark), at 37°, 50% CO₂ in a humidified atmosphere (as described in Quarterly Report #6). Conditioned medium of F-10 was exchanged every 48 hours, for a follow up period of 5 days. Samples for PGE₂, LTB₄ and IL-1 were taken before medium exchange on days 1, 3 and 5.

EXPERIMENTAL DESIGN

Two groups of cultured cells were involved in the study:

- 1) Control group grown in F-10 medium.
- 2) bFGF treated group - cells grown in F-10 medium containing 10 μ g/ml bFGF.

RESULTS

Our results (Table 1) indicate that on day 1 post seeding PGE₂ levels in control did not differ significantly from those of bFGF treated cultured RPE cells. However, bFGF addition to medium caused a initially significant ($p < 0.05$) increase in LTB₄ levels (on day 1), although at later stages LTB₄ levels in the bFGF group did not differ significantly from the corresponding control values.

LTB₄ values over a 5 day follow - up in the two groups did not change significantly as compared with the significant decrease in PGE₂ levels. The latter demonstrated an initial peak on day 1, while at the later stages its levels were undetectable.

Table 1: Changes in prostaglandin E₂, leukotriene B₄ and Interleukin in cultured RPE following the addition of basic Fibroblastic Growth Factor

| | | Day 1 | Day 3 | Day 5 |
|--------------------------------------|---------|-------|-------|-------|
| Prostaglandin E ₂ (pg/ml) | Control | 140 | < 50 | < 50 |
| | FGF | 175 | < 50 | < 50 |
| Leukotriene B ₄ (pg/ml) | Control | 38.7 | 42.5 | 51.2 |
| | FGF | 55.0 | 38.7 | 55.0 |
| Interleukin-1- α (pg/ml) | Control | 00.0 | 6.76 | 3.95 |
| | FGF | 0 | 6.89 | 2.36 |

Thus, the presence of bFGF in the conditioned media did not affect the natural course of PGE₂ and LTB₄ production by cultured RPE. Another observation is that longevity of culturing did not have an effect on the amounts of PGE₂ and LTB₄ produced by RPE cells. This finding is noteworthy, since the number of cells in each well was growing during the follow - up.

It is our assumption that the initial peak of PGE₂ arouses as a result of an initial trauma post seeding, while later in the course of continuous growth in the right conditioned medium no stimulation for production of excessive LTB₄ or PGE₂ exists. Additionally, since in our model bFGF also had an effect on both metabolites only during the 1st day, this might represent the "traumatized" cellular response rather than a response to bFGF.

Interleukin 1- α changes in control and bFGF groups were similar; thus, on day 1, IL-1 was undetectable, while peak values, similar in both groups, were noted on day 3. The significance of IL-1 α decrease on day 5 is still to be considered.

Histology - The infiltration of inflammatory cells at the cornea with the lasered pellet is another indication to the fact that angiogenesis is closely related to an inflammatory activity (Fig 12).

CONCLUSIONS

PGE₂ levels were high on the 1st day following RPE seeding, both in control and in bFGF treated groups. Later their levels decreased in the two groups and bFGF was found to have no effect on induction of excessive PGE₂ production. PGE₂ excessive levels during the 1st day post seeding might be indicative of trauma associated with prior handling.

LTB₄ levels were elevated by the addition of bFGF but only on the 1st day. Later changes over a 5 day period were negligible. Similarly, IL-1 α levels were not affected by the bFGF treatment when compared to control.

This pilot study points to the fact that the known ability of RPE to produce cytokinins was not significantly affected by the addition of bFGF.

Further extension of this study is required to elucidate and confirm these findings.

Conclusions - 1

In our study we found the following:

- 1) Vitreous obtained from lasered eyes possess an angiogenic activity in rat cornea. Angiogenesis is associated with an inflammatory reaction.
- 2) Angiogenesis is affected by a superoxide inhibitor D.P.I which inhibits the NADPH oxidative system.
- 3) Vitreous of lasered eyes increases the all proliferation of vascular endothelium cells in a dose dependent way. RPE cells and fibroblasts are also affected by either intact or lasered vitreous, but each cell line has a different pattern of reaction.

Conclusions - 2

This pilot study, added to the debate on the proliferative capacity of intact vitreous, confirms data showing induction of cellular proliferation of RPE in-vitro by intact vitreous (1-2). Our data show that lasered vitreous capacity to induce enhanced RPE cell proliferation is greater than that of intact vitreous or FGF.

The effect of intact or lasered vitreous on vascular endothelium follows a pattern different from that seen in RPE cell culture:

- 1) intact vitreous does not induce a significant proliferation in that cell line;
- 2) lasered vitreous, when used in low concentrations, induces a two-fold increase in cell division;
- 3) lasered vitreous in higher concentration exerts an inhibitory effect on vascular cell division.

Our findings are unique, as no similar data have been reported so far. They point to the fact that mediators present in the lasered vitreous have a dual effect so that lower concentrations elicit a substantial proliferation. This finding might be of significant clinical importance.

Conclusions - 3

Further studies should elucidate the role of "free radicals" during the neovacuogenic.

The finding on dose dependent proliferative effect of lasered vitreous on RPE and vascular endothelium is unique and might explain the formation of late scarring post laser exposure. Preventive measures should be studied so as the least scarring will occur, especially by use of anti oxidant therapy.

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Fig. 1

$[^3\text{H}]$ -Thymidine incorporation to
Endothelial ABAE cells
effect of lasered vitreous

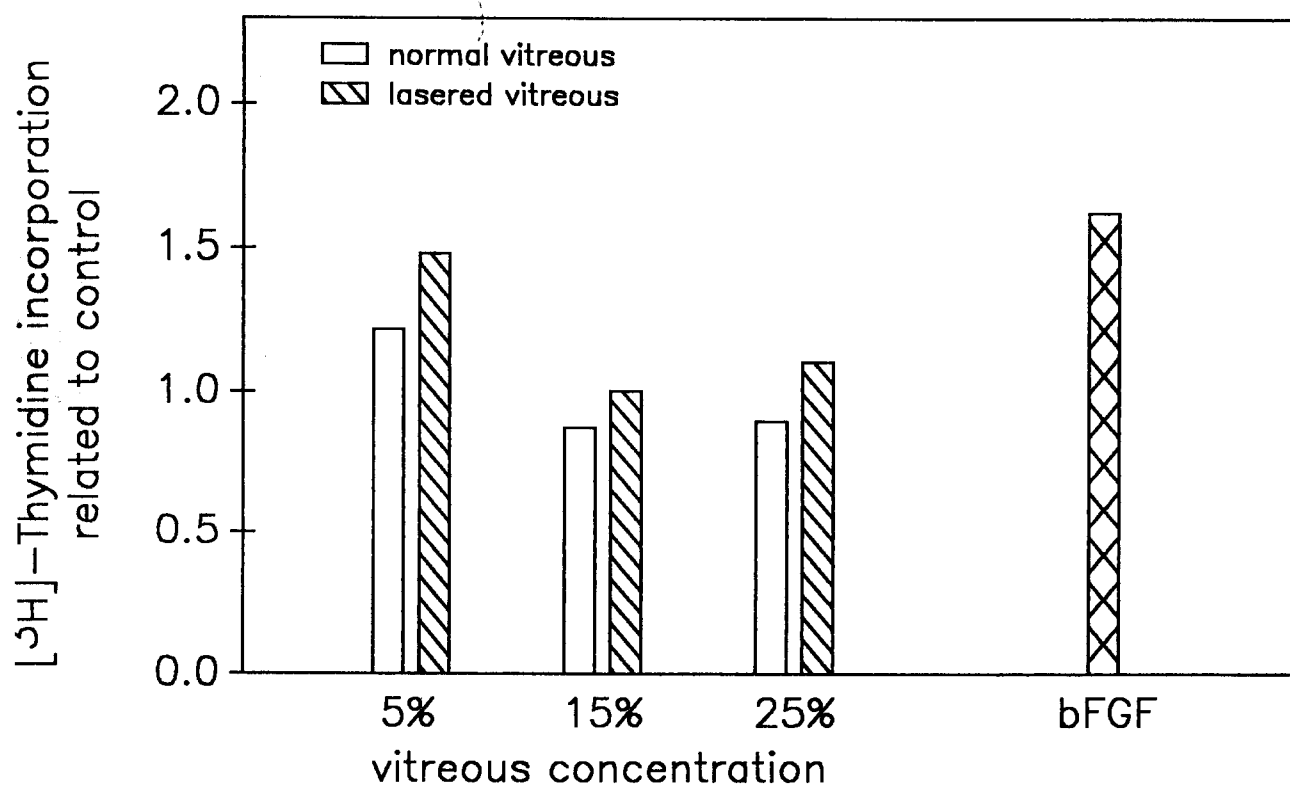


Fig. 2

[³H]–Thymidine incorporation to
Endothelial ABAE cells
effect of lasered vitreous

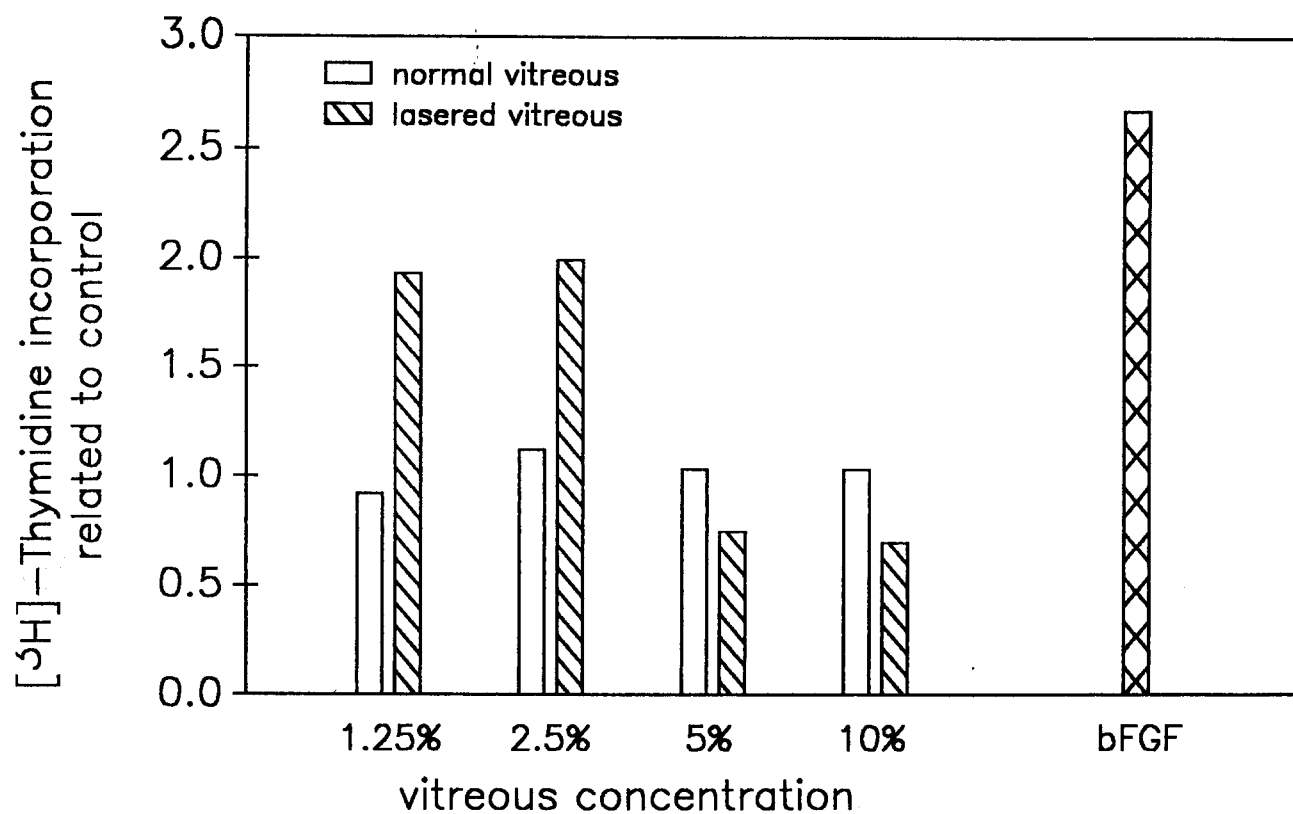


Fig. 3

[³H]–Thymidine incorporation to
human Retinal Pigment Epithelial cells
effect of lasered vitreous

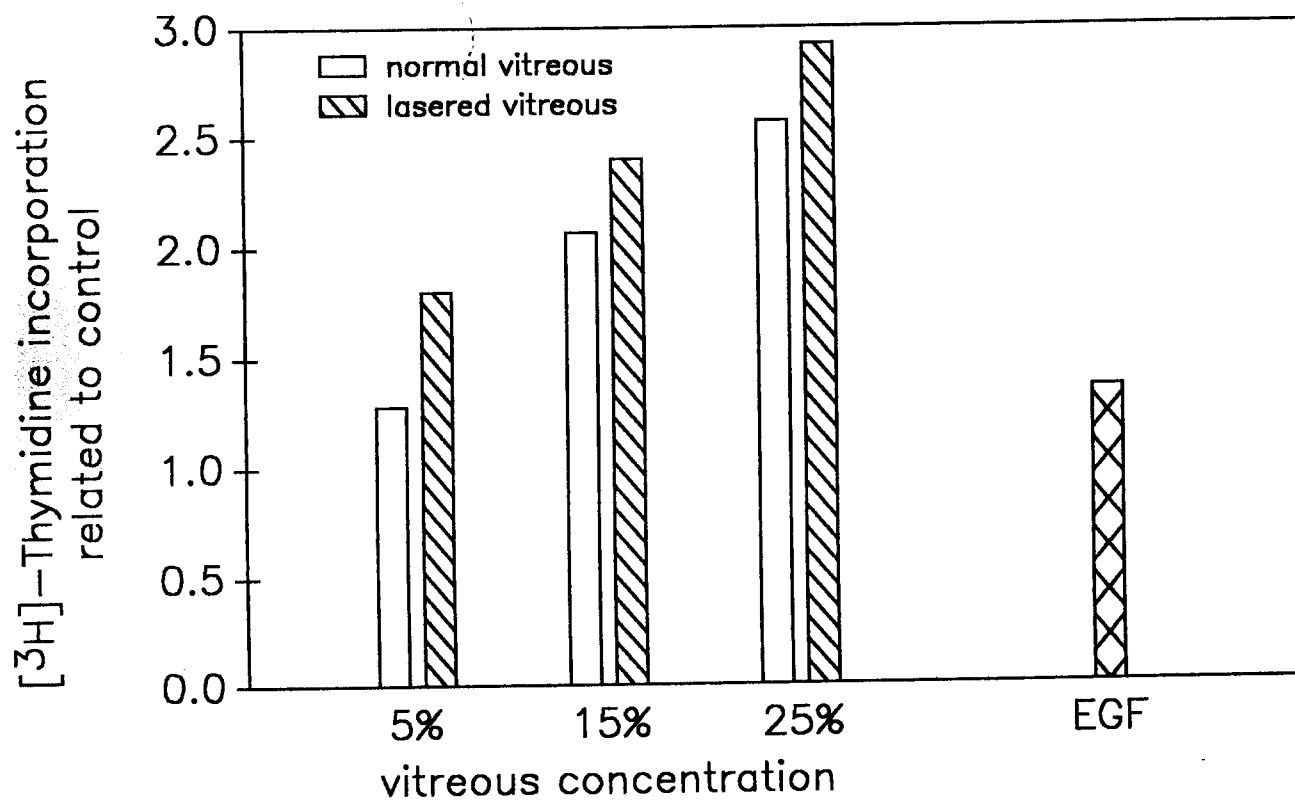


Fig. 4

[³H]–Thymidine incorporation to
Human Skin Fibroblast cells
effect of lasered vitreous

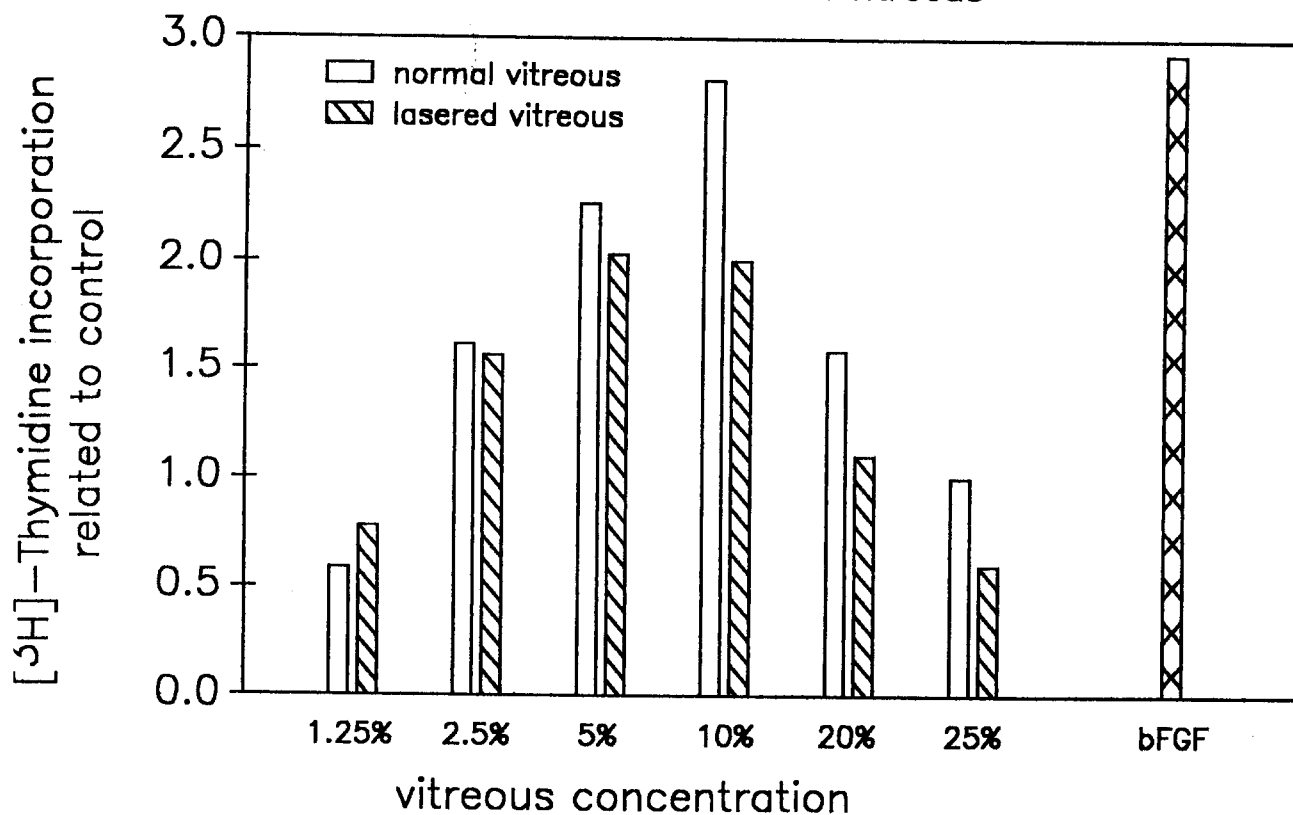


Fig. 5 Initial limbal congestion — comparison of control groups

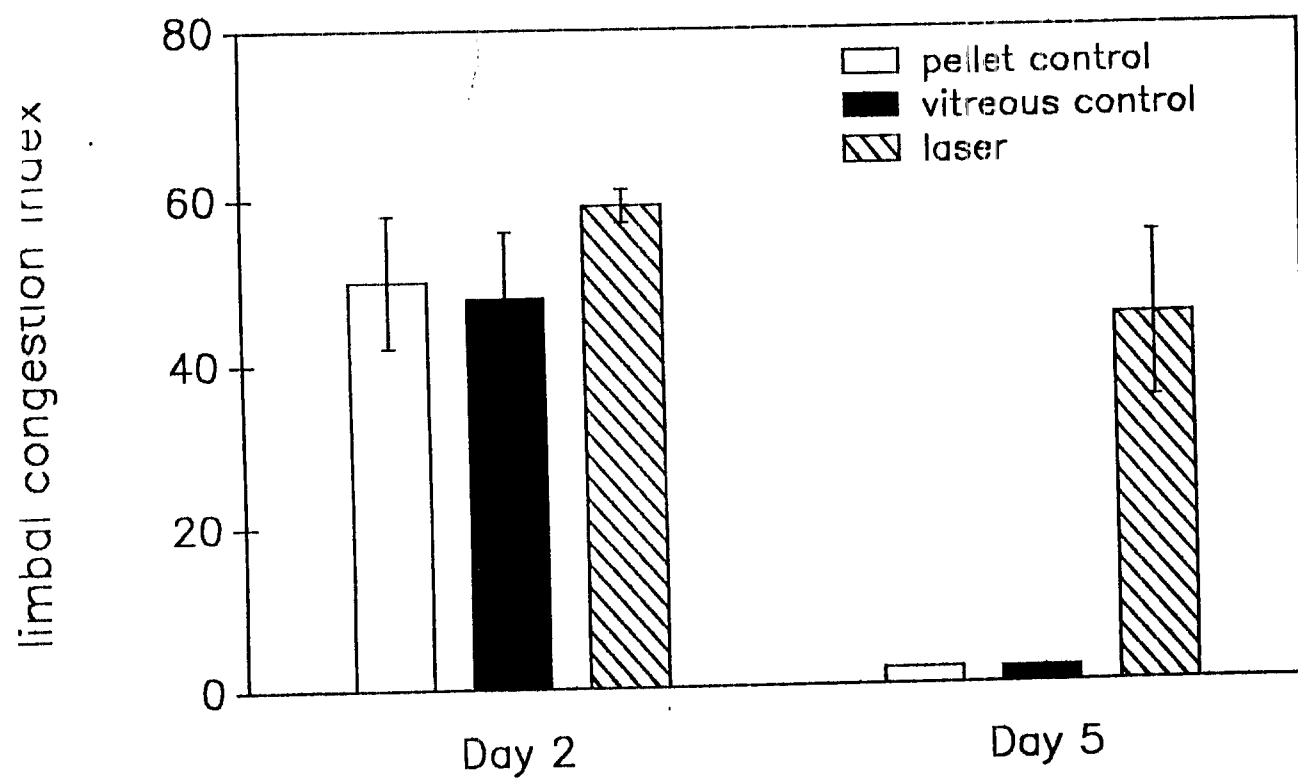


Fig. 6 Limbal vasodilatation of collateral vessels
in cornea containing extract of lasered eyes

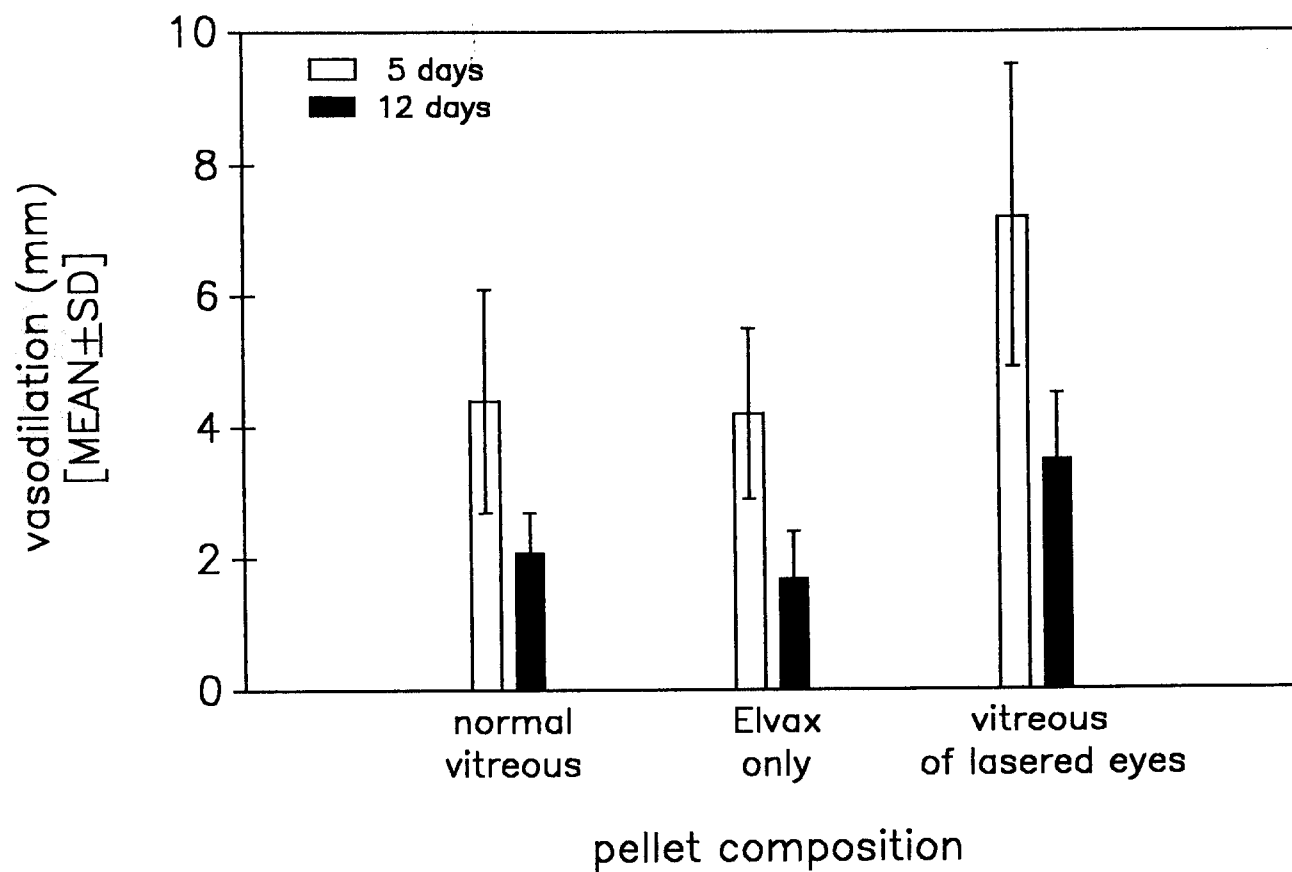
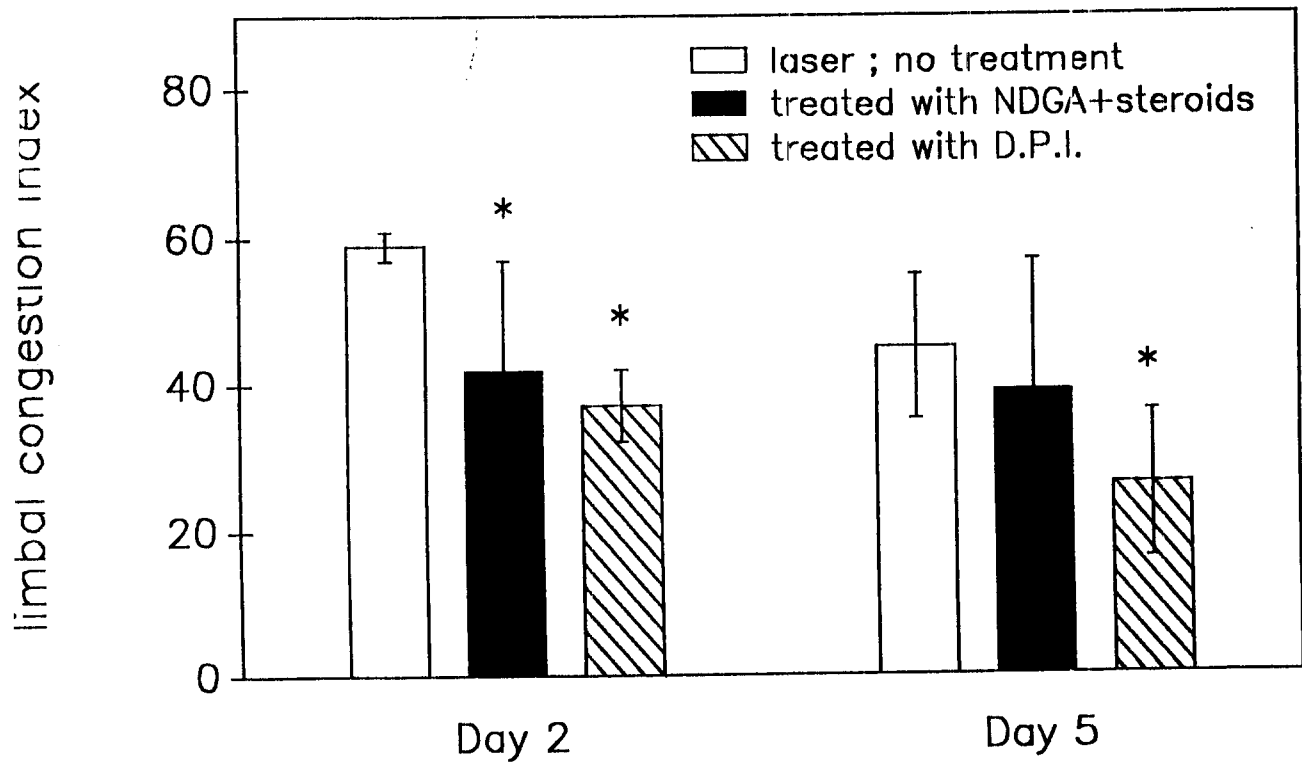


Fig. 7

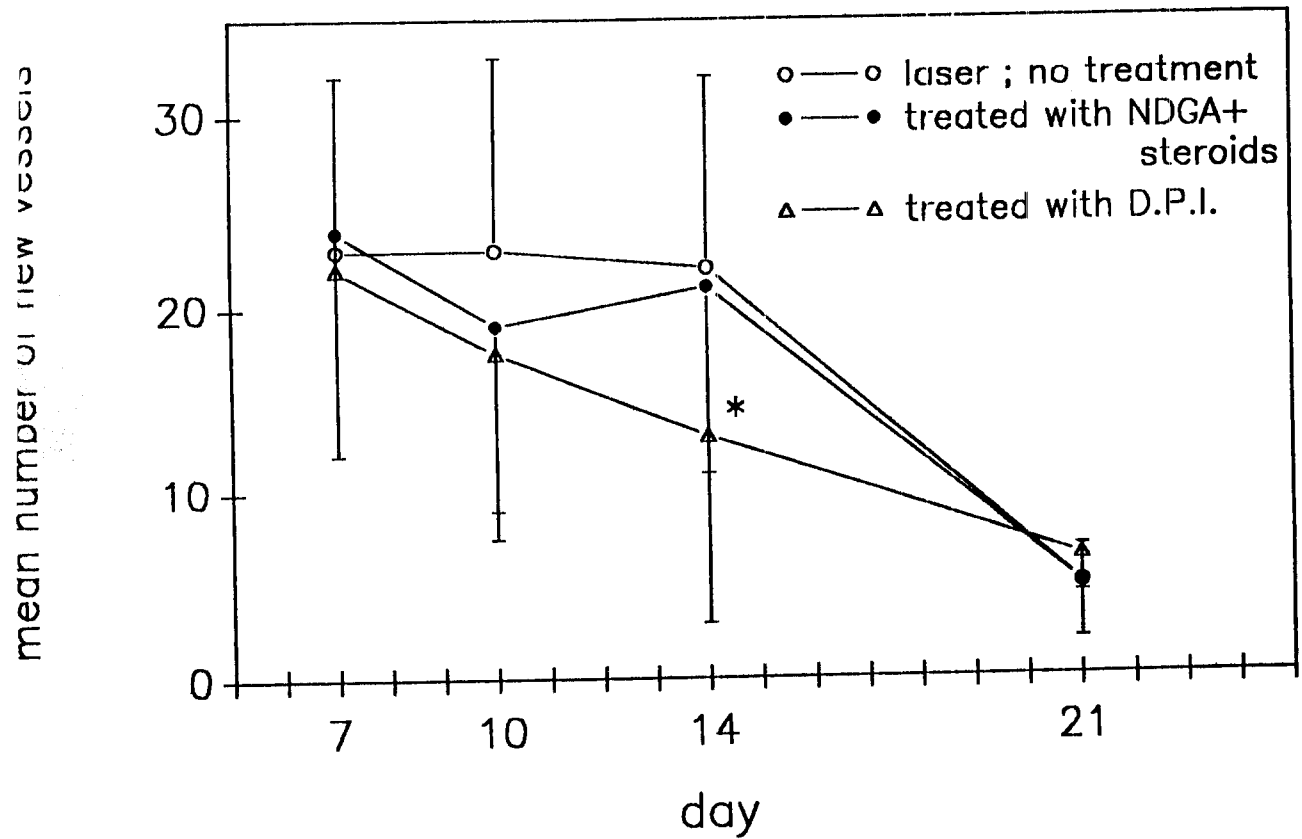
Initial limbal congestion – the effect of treatment



* $p < 0.05$

Fig. 8

Mean number of newly formed blood vessels
in a pellet implanted rat's cornea—
The effect of various treatments during late phase



* $p < 0.05$

Fig. 9

Budding of collateral vessels
in cornea containing extract of lasered eyes –
effect of anti-oxidant treatment

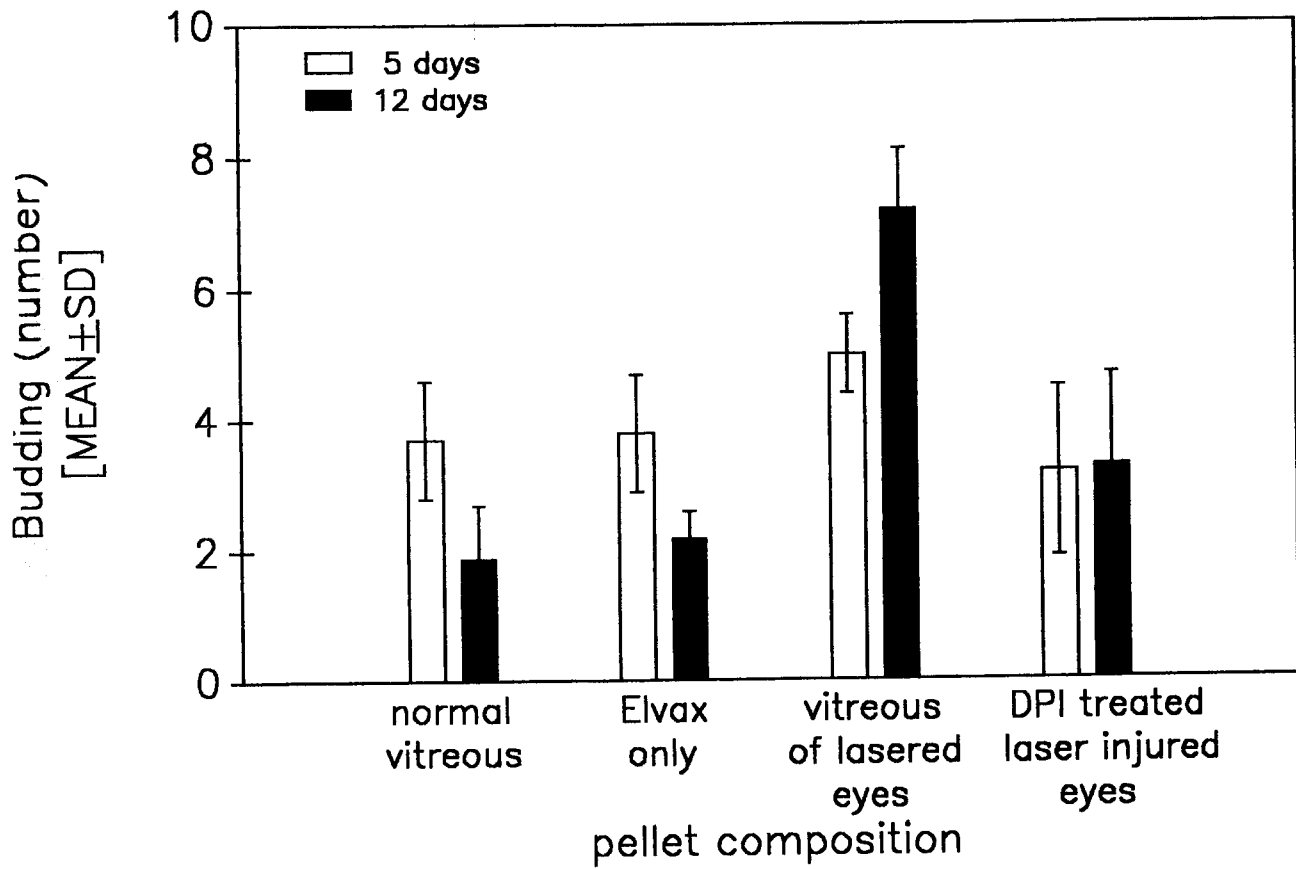


Fig. 10 Length of newly formed blood vessels
in cornea containing extract of lasered eyes –
effect of anti-oxidant treatment

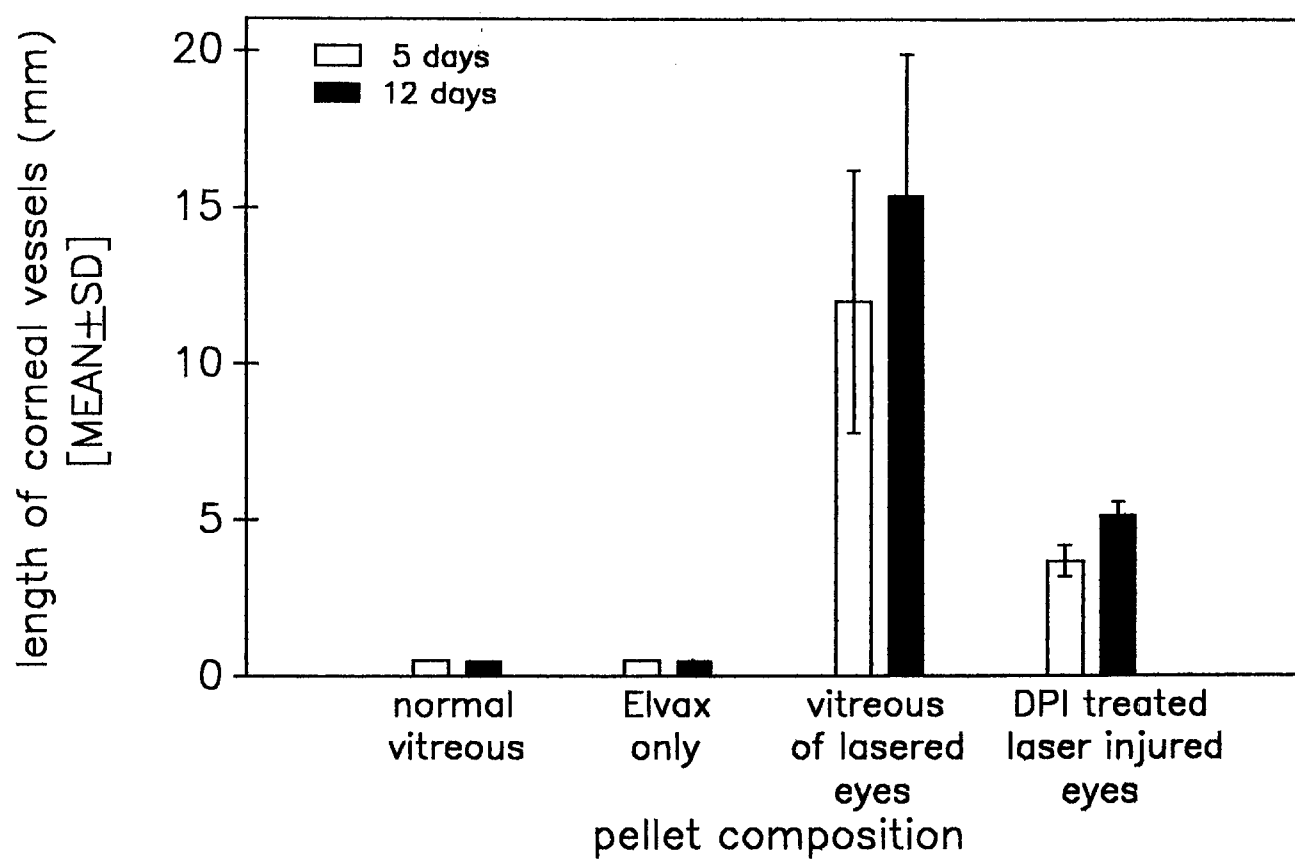


Fig. 11 Inflammatory cell infiltration in cornea containing vitreous of laser injured eyes

